



# Antibacterial and antimycobacterial activities of South African *Salvia* species and isolated compounds from *S. chamelaeagnea*

G.P.P. Kamatou<sup>a</sup>, S.F. Van Vuuren<sup>a</sup>, F.R. Van Heerden<sup>b</sup>, T. Seaman<sup>c</sup>, A.M. Viljoen<sup>d,\*</sup>

<sup>a</sup> Department of Pharmacy and Pharmacology, Faculty of Health Sciences, University of the Witwatersrand, 7 York Road, Parktown 2193, South Africa

<sup>b</sup> School of Chemistry, University of KwaZulu-Natal Pietermaritzburg, Private Bag X01, Scottsville 3209, South Africa

<sup>c</sup> Division of Pharmacology, Faculty of Health Sciences, University of Cape Town, South Africa

<sup>d</sup> School of Pharmacy, Tshwane University of Technology, Private Bag X680, Pretoria 0001, South Africa

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## Abstract

Extracts of 16 South African *Salvia* species commonly used in traditional medicine to treat various microbial infections were investigated for *in vitro* antibacterial and antimycobacterial activities using the micro-dilution and respiratory BACTEC method, respectively. The micro-organisms tested include two Gram-positive (*Staphylococcus aureus* and *Bacillus cereus*); two Gram-negative (*Escherichia coli* and *Klebsiella pneumoniae*) bacterial strains and the common pathogen responsible for tuberculosis, *Mycobacterium tuberculosis*. Extracts of the majority of species exhibited moderate to good antibacterial activity with minimum inhibitory concentration (MIC) values ranging from 0.03 to 8.00 mg/ml. Promising activity was observed against *M. tuberculosis* (MIC  $\leq$  0.50 mg/ml) with *S. radula*, *S. verbenaca* and *S. dolomitica* displaying the most favourable activity (MIC: 0.10 mg/ml). The antibacterial bioassay-guided fractionation of *S. chamelaeagnea* resulted in the isolation of four compounds: carnosol, 7-*O*-methylepirosmanol, oleanolic acid and its isomer ursolic acid as the active principles against *S. aureus*. The *in vitro* antibacterial and antimycobacterial activities may support the use of *Salvia* species in traditional medicine to treat microbial infections.

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## 1. Introduction

Despite the progress in understanding the growth and control of many pathogens, nearly all the diseases affecting millions of people (especially in developing countries) are still caused by micro-organisms. Tuberculosis (TB), a mycobacterial infection, remains a serious health problem in many regions of the world. It is estimated that a third of the world's population is infected with *Mycobacterium tuberculosis* and approximately 1.7 million people have died from TB worldwide in 2003 (WHO, 2005). In 2004, TB killed half a million people in Africa, mostly young men and women in their most productive years. Africa is the only continent where the TB rates are increasing. The current threat in TB treatment

lies in the emergence of resistant strains (Mativandlela et al., 2006). In the past years, the development of resistance by pathogens to many of the commonly used antibiotics provides sufficient impetus for further attempts to search for new antimicrobial agents (Grange and Davey, 1990). Plant-derived natural products may offer potential leads for novel compounds and species of the genus *Salvia* (sage) are potential candidates in this regard, since they have long been used as a frequent component of herbal mixtures to treat tuberculosis and a range of microbial infections (Watt and Breyer-Brandwijk, 1962).

The genus *Salvia* belongs to the family Lamiaceae and encompasses about 900 species worldwide of which 26 are found in southern Africa (Jäger and Van Staden, 2000). Members of the genus are extensively used in South Africa in healing rites especially to treat infections. This work forms part of a greater study to document the chemistry and biological activity of South African medicinal aromatic plants.

\* Corresponding author.

E-mail address: [viljoenam@tut.ac.za](mailto:viljoenam@tut.ac.za) (A.M. Viljoen).

## 2. Materials and methods

### 2.1. Plant material

The aerial parts of 16 *Salvia* species were collected at various localities in South Africa between December 2003 and December 2004, predominantly from the Cape region (Table 1). Variation within and between populations were not taken into account and only one representative per species was considered. The identity of each species was confirmed by the South African National Biodiversity Institute (Pretoria) and voucher specimens were deposited in the School of Pharmacy, Tshwane University of Technology, Pretoria, South Africa.

### 2.2. Chemicals, reagents and standards

Acetic acid, dioxane and DMSO were obtained from Saarchem; analytical grade of acetone, chloroform, dichloromethane, ethyl acetate, hexane, methanol and toluene were from Rochelle Chemicals; Tryptone Soya agar (TSA) and Tryptone Soya broth (TSB) were purchased from CA Milsch; ciprofloxacin was obtained from Merck and *p*-iodonitro-tetrazolium chloride (INT), ursolic acid (042k1240) and oleanolic acid (093k0961) were from Sigma. The BACTEC™ 12B medium and rifampicin were obtained from Becton Dickinson. Column chromatography was carried out on glass columns with silica gel 60, 70–230 mesh ASTM. Thin-layer chromatography (TLC) was done on pre-coated silica gel 60 F254 plates (0.25 mm, Macherey-Nagel).

### 2.3. Plant extraction

Aerial parts of each plant were air-dried at room temperature and the finely ground powder extracted three times with methanol: chloroform (1:1, v/v) in a 36–40 °C water bath for 3 h. The suspension was filtered through Whatman® No 1 filter paper and the filtrate was concentrated to dryness under reduced pressure.

### 2.4. Determination of the antibacterial and antimycobacterial activities

The *in vitro* antibacterial activity was evaluated by the micro-dilution assay (Eloff, 1998) against two Gram-positive organisms *Bacillus cereus* (ATCC 11778) and *Staphylococcus aureus* (ATCC 25923); and, two Gram-negative organisms *Klebsiella pneumoniae* (NTCC 9633) and *Escherichia coli* (ATCC 8739). Briefly, the stock solution (64 mg/ml in acetone) was serially diluted (1:1) with sterile water in a 96-well microtitre plate before an equal volume (100 µl) of bacterial suspension yielding approximately an inoculum size of  $1 \times 10^6$  CFU/ml added to the wells and the plate incubated for 24 h. Thereafter, 0.04% (w/v) INT was added to each well and the plate left at room temperature for 6 h before the results were recorded. Sterile broth containing bacterial suspension was used to observe the normal growth, while ciprofloxacin was used as positive antibacterial control.

The susceptibility of *M. tuberculosis* H37Ra (ATCC 25177) to the plant extracts was assayed using the radiometric technique (Siddiqi et al., 1981). The mycobacterial suspension

Table 1  
The antibacterial and antimycobacterial activity of indigenous *Salvia* species against various pathogens

Species/isolated compounds	Voucher number	Antibacterial activity (MIC values in mg/ml)				Antimycobacterial activity			
		<i>E. coli</i> (ATCC 8739)	<i>K. pneumoniae</i> (NCTC 9633)	<i>B. cereus</i> (ATCC 11778)	<i>S. aureus</i> (ATCC 25923)	<i>M. tuberculosis</i> H37Ra ΔGI values (mg/ml)			MIC values (mg/ml)
						1.0	0.5	0.1	
<i>S. africana-caerulea</i>	AV 875	4.00	4.67	6.00	3.00	0 (S)	–3 (S)	73 (R)	0.50
<i>S. africana-lutea</i>	AV 873	3.00	3.00	0.75	0.75	0 (S)	0 (S)	50 (R)	0.50
<i>S. albicaulis</i>	AV 894	4.00	4.67	1.00	1.00	0 (S)	0 (S)	41 (R)	0.50
<i>S. aurita</i>	AV 1066	4.00	4.00	0.25	0.05	0 (S)	4 (S)	33 (R)	0.50
<i>S. chamelaeagnea</i>	AV 848	1.00	3.00	0.03	0.06	0 (S)	1 (S)	37 (R)	0.50
<i>S. disermas</i>	AV 1194	4.00	2.00	3.00	0.75	0 (S)	18 (S)	520 (R)	0.50
<i>S. dolomitica</i>	AV 838	3.33	2.00	0.75	0.03	2 (S)	–1 (S)	11 (S)	0.10
<i>S. garipensis</i>	AV 1193	1.88	2.81	0.75	4.00	nd	nd	nd	nd
<i>S. lanceolata</i>	AV 877	4.00	2.00	3.00	2.00	0 (S)	–1 (S)	40 (R)	0.50
<i>S. muirii</i>	AV 874	2.00	3.00	0.25	0.36	0 (S)	7 (S)	33 (R)	0.50
<i>S. radula</i>	AV 880	>16.00	4.00	1.00	0.06	–1 (S)	4 (S)	10 (S)	0.10
<i>S. repens</i>	AV 615	2.00	3.00	0.03	0.25	0 (S)	2 (S)	48 (R)	0.50
<i>S. runcinata</i>	AV 679	2.00	4.00	0.03	0.25	0 (S)	4 (S)	40 (R)	0.50
<i>S. schlechteri</i>	AV 1068	4.00	3.00	0.75	0.25	0 (S)	19 (S)	38 (R)	0.50
<i>S. stenophylla</i>	AV 893	4.00	2.00	0.03	0.06	0 (S)	8 (S)	38 (R)	0.50
<i>S. verbenaca</i>	AV 631	8.00	2.00	2.00	3.00	–2 (S)	3 (S)	5 (S)	0.10
Carnosol		2.00	2.00	0.02	0.03	–	–	–	–
Oleanolic acid		3.41	1.71	1.28	3.75	–	–	–	–
Ursolic acid		2.50	3.75	$7.32 \times 10^{-3}$	0.01	–	–	–	–
7-O-Methyl-epirosmanol		nd	nd	0.01	0.02	–	–	–	–
Controls		$4.00 \times 10^{-5a}$	$1.60 \times 10^{-4a}$	$4.10 \times 10^{-5a}$	$3.10 \times 10^{-4a}$	0 (S) <sup>b</sup>	0 (S) <sup>b</sup>	0 (S) <sup>b</sup>	$<2.00 \times 10^{-3b}$

KBG: Kirstenbosch Botanical Garden, S: sensitive; R: resistant. ΔGI value of the control vial 29, 29 and 28 for 1, 0.5 and 0.1 mg/ml, respectively. For the sample to be active against *M. tuberculosis*, ΔGI must be less than the ΔGI of the control vial. <sup>a</sup>Antibacterial control ciprofloxacin, <sup>b</sup>anti-tuberculosis rifampicin control vial (2 µg/ml) was included in each run. nd: not determined due to insufficient amount of the sample.

was adjusted to approximately match that of McFarland No 1 turbidity standard (Lall and Meyer, 1999). A 12B vial was then inoculated with 100  $\mu$ l of the mycobacterial suspension and incubated at 37 °C until the growth reached 400–500. The suspended culture was used undiluted to inoculate a set of 12B vials, 100  $\mu$ l per vial yielding approximately  $1 \times 10^4$ – $1 \times 10^5$  CFU/ml (Heifets et al., 1985) before the filtered stock solution of plant extract (100  $\mu$ l, 42 mg/ml in 50% methanol) was added to the vials to obtain final concentrations of 1, 0.5 and 0.1 mg/ml. The control vial was inoculated with a 1:100 dilution of the culture and the vials were incubated at 37 °C and monitored on a daily basis. Rifampicin, a standard antituberculosis drug, was used as a positive control at a final concentration of 2  $\mu$ g/ml.

The lowest concentration of the test sample in which no growth occurred was defined as the minimum inhibitory concentration (MIC). To evaluate the antimycobacterial activity, the difference in the growth index (GI) values of the last two days was designated as  $\Delta$ GI and the results were interpreted once the control vial reached a GI value 30. The following formula was used to interpret the results:  $\Delta$ GI control >  $\Delta$ GI extract = resistant;  $\Delta$ GI control <  $\Delta$ GI extract = susceptible and the MIC was determined as the lowest concentration of drug inhibiting more than 99% of the mycobacterial population. For each analysis, the treatment was performed singularly and at least three experiments were carried out.

### 2.5. Isolation and identification of antibacterial compounds from *S. chamelaeagnea*

To determine the compounds responsible for the antibacterial activity, *S. chamelaeagnea* was selected as it showed good activity. Briefly, dried aerial parts (423.4 g) were powdered, extracted with methanol:chloroform (1:1 ratio, v/v) and evaporated under reduced pressure to yield 19.6 g. The residue was subjected to column chromatography with silica gel 60 using hexane:dichloromethane (9:1), dichloromethane:methanol (6:1) and 100% methanol consecutively as eluent. Spots on TLC plates were visualized under UV light and sprayed with anisaldehyde–H<sub>2</sub>SO<sub>4</sub> reagent followed by heating at 100 °C for 5 min and fractions showing similar chromatograms were combined to yield five main fractions. To identify the active principles, a direct antibacterial bio-assay (Dilaka and Meyer, 1996) on a TLC plate was employed using the agar-overlay method with *S. aureus* as the test organism. Each of the five fractions (1–5) collected was spotted on a TLC plate. The dried TLC plate was then sterilized under UV light (254 nm) for 1 h before being placed in a petri dish containing the solidified agar. The UV light was used to sterilize the dried extract instead of autoclaving in order to avoid destruction of the potency of the active constituents by heat (Okoli and Iroegbu, 2004). The plate was then sprayed with *S. aureus* culture in Tryptone Soya agar and left to solidify. After 1 h in the fridge (to allow compounds on the TLC to diffuse normally), the plate was removed and incubated at 37 °C. At the end of the overnight incubation, it was sprayed with a 0.04% aqueous solution of INT to visualize the inhibition zone. The inhibition zone was indicated by the

clear zone on the red background. Only fraction 4 exhibited activity and was selected for further analysis. It was evaporated under vacuum to yield 6.21 g. This fraction was rechromatographed in a small column (2.5 cm  $\times$  45 cm) using hexane:ethyl acetate:acetic acid (12:3:0.02) as an eluent. A total of 322 subfractions in 3 ml test tubes were collected and compound 1 (120 mg) was obtained as white crystals. Similar fractions were then combined and subjected to a bio-autographic assay on a TLC plate to detect a fraction containing other active compound (s). The eluted fraction was monitored on a TLC plate developed with the same solvent system. Pooled fractions were concentrated with a rotary evaporator and rechromatographed using the same column with hexane:ethyl acetate:acetic acid (12:2:0.01) as the solvent system. The combined fractions yielded seven subfractions and only subfractions 2 and 4 showed activity. Subfraction 2, eluted with hexane:ethyl acetate:acetic acid (12:3:0.01) and toluene:ethyl acetate (12:3) successively after repeated column chromatography yielded compound 2 (12 mg). Compounds 3 and 4 (7 mg) were obtained as an inseparable mixture after repeated column chromatography of subfraction 4 with hexane:ethyl acetate:acetic acid (12:3:0.01) followed by toluene:ethyl acetate (12:3). The isolated compounds were structurally characterized using <sup>1</sup>H and <sup>13</sup>C, nuclear magnetic resonance spectroscopy (NMR), mass spectrometry (MS) on the GC-MS QP 2010 gas chromatography mass spectrometer and the structures compared to those found in literature [1 and 2 (Urones et al., 1998), 3 and 4 (Mahato and Kundu, 1994)].

## 3. Results and discussion

### 3.1. Antibacterial and antimycobacterial activities

The results of the antibacterial activity showed that the methanol: chloroform (1:1) extracts exhibited good to moderate antibacterial activity with MIC values ranging between 0.03 to 8.00 mg/ml (Table 1). Only *S. radula* failed to inhibit the growth of *E. coli* but showed significant activity against the other pathogens. The most promising activity was obtained with *S. chamelaeagnea*.

The results for the antimycobacterial activity were interpreted after two weeks when the control vial containing 1:100 dilution of the inoculum reached a  $\Delta$ GI value of approximately 30. The  $\Delta$ GI results and the MIC values of the species examined are listed in Table 1. The *M. tuberculosis* was sensitive to all extracts and therefore exhibited potent antimycobacterial activity. Of the species tested, 11 have MIC values of 0.50 mg/ml, while the MIC values of three species are 0.10 mg/ml. It was noted that species such as *S. verbenaca*, *S. radula* and *S. dolomitica* which displayed good antimycobacterial activity (MIC: 0.10 mg/ml) also exhibited good antibacterial activity. According to Fabry et al. (1998), the solvent extracts of plants with MIC values less than 8 mg/ml may be considered as antimicrobially active. The values obtained in this study were less than 8.0 mg/ml against all the pathogens tested. These results are consistent with the pattern for the *in vitro* activity emerging from published works. In our previous investigation (Kamatou et al., 2005), we found that

*S. runcinata*, *S. repens* and *S. stenophylla* were also active against a range of pathogens. The antibacterial activity of exotic *Salvia* species has also been reported in the literature (Lee et al., 1999; Ulubelen et al., 2001; Tepe et al., 2004). *Salvia* species are rich in phenolic compounds which are known to display a broad range of biological activities such as antibacterial, anti-oxidant, anticancer and anti-inflammatory activities (Lu and Foo, 2001).

Infections caused by *S. aureus* are among the most difficult to treat with standard clinical antibacterial agents (Tomás-Barberán et al., 1990). The growth of *S. aureus* was inhibited by all extracts (Table 1) especially by *S. radula*, *S. chamelaeagnea*, *S. aurita* and *S. dolomitica* ( $\text{MIC} \leq 0.06$  mg/ml). Thus, these plants could yield compounds that may improve the treatment of infections caused by *S. aureus* in particular. It was reported that many species of *Salvia* are used in decoctions in South Africa by Sotho people against various bacterial infections such as throat inflammation, colds and coughs and to treat pulmonary diseases such as tuberculosis (Watt and Breyer-Brandwijk, 1962). The positive *in vitro* results indicate that *Salvia* species may be used to combat microbial infections.

### 3.2. Elucidation and identification of the isolated compound from *S. chamelaeagnea*

The methanol:chloroform extract which exhibited the best antibacterial activity was subjected to column chromatography, isolation and purification to yield four known compounds. The compounds were identified by analysis of their NMR and MS data and comparison with data reported in the literature. Compound 1, isolated as white crystals, was a diterpene characterized as carnosol. Compound 2, also a diterpene, was isolated as a white powder and characterized as 7-*O*-methylepirosmanol. Compounds 3 and 4 were isolated as an inseparable mixture of

triterpenes identified as (3 $\beta$ )-3-hydroxyurs-12-en-28-oic acid, commonly known as ursolic acid ( $\text{C}_{30}\text{H}_{48}\text{O}_3$ ), and its isomer (3 $\beta$ )-3-hydroxyolean-12-en-28-oic acid, generally known as oleanolic acid. The structures of the isolated compounds are presented in Fig. 1. The antibacterial MIC values of the isolated compounds were also determined (Table 1). Ursolic acid exhibited the most favourable activity among the isolated compounds. It was noted that this compound was 175 times more active than its isomer oleanolic acid against Gram-positive bacteria.

The isolated compounds exhibited some degree of activity against *S. aureus* and to a lesser degree against *B. cereus*, while no clear pattern was obtained between the isolated compounds and the crude extract against other pathogens. The solvent extracts are crude preparations and it was expected that the isolated compounds would display higher antibacterial activity than the crude extract, as it would be present in a higher concentration. However, this was not the case particularly for oleanolic acid and may partly be attributed to the liability of the isolated compounds. Furthermore, it is not possible to quantify the antibacterial activity from a bio-autographic assay where the inhibition spot is by nature a positive/negative response and does not reveal detail about the degree of activity of a compound. The mechanism of many phytomedicines is still unknown and there are several instances where the total herb extract exhibits a better effect than an equivalent dose of an isolated compound. The anti-inflammatory activity of ginkgolides isolated from *Ginkgo biloba* was less effective than the plant extract (Williamson, 2001). Horiuchi et al. (2007) also found that a crude extract from *Salvia officinalis* reduced the MICs of aminoglycosides in vancomycin-resistant enterococci, while the effective compound from the extract (carnosol) showed weak antimicrobial activity. However, the effect of

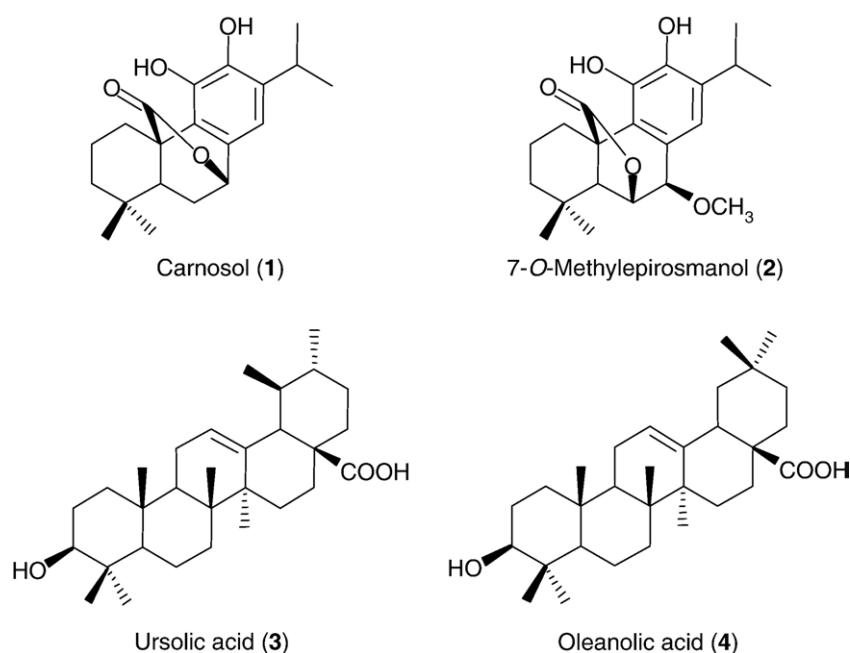


Fig. 1. Chemical structures of the isolated compounds.



carnosol and carnosic acid with gentamicin was synergistic. Oluwatuyi et al. (2004) also found that incorporation of carnosol and carnosic acid into the growth medium caused a 32- and 16-fold potentiation of the activity of erythromycin against an erythromycin effluxing strain *S. aureus*. Since four compounds were isolated, it is possible that they may also act synergistically to give the overall antibacterial activity obtained with the crude extract.

Carnosol has previously been reported to displayed antibacterial activity against various micro-organisms including *S. aureus*, *B. subtilis*, *E. coli* and *C. albicans* (Collins and Charles, 1987; Dimayuga et al., 1991; Oluwatuyi et al., 2004). It is a compound present in many *Salvia* species and was first isolated from *S. carnososa* (Dougl.) (White and Jenkins, 1942). Since then, this compound has been detected in *Rosmarinus officinalis* and many other species belonging to the Lamiaceae (Wu et al., 1982). A mixture of ursolic acid and its isomer oleanolic acid was isolated previously from *Swertia arisanensis* (Gentianaceae) (Lin et al., 1987) and the two compounds have been found in more than 120 plant species including the Lamiaceae. Contemporary scientific research which led to the isolation and identification of ursolic acid revealed and confirmed that several pharmacological effects, such as antitumour, hepatoprotective, anti-inflammatory, anti-ulcer, antimicrobial, antihyperlipidemic and antiviral activity can be attributed to ursolic acid (Liu, 1995). Ursolic acid/oleanolic acid and derivatives from the Lamiaceae exhibited antimicrobial activity and these therapeutic effects have been confirmed by laboratory testing (Sattar et al., 1995). Ursolic acid was also identified as one of the active principles in *Rosmarinus officinalis* (Lamiaceae) to inhibit the growth of some food borne pathogens (Collins and Charles, 1987).

The potency of natural substances could provide a scientific basis for some of the health benefits claimed for indigenous sages in folk medicine and warrant further studies to assess their potential as effective natural remedies. The bioassay-guided fractionation revealed four compounds responsible for the antibacterial activity from *S. chamelaeagnea*, however, further investigation is required in order to determine the compounds responsible for the antimycobacterial activity from the most active species and to evaluate the activity of isolated compounds in combination. Carnosol and ursolic acid inhibit the growth of food spoilage microbes. As carnosol is known to possess good anti-oxidant activity, this compound may prove a useful substitute for some of the phenolic anti-oxidants in current use, while offering an improved inhibitory capacity to microbial growth. Most of the phytomedicines are on the drug market as whole extracts of plants and traditional practitioners have always believed that the collective action of many compounds present in a plant is a vital part of the therapeutic efficacy.

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